



ISOLATION AND SEQUENCING OF *CYP19* GENE (EXON9) IN EGYPTIAN BUFFALOES

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ABSTRACT

Numerous studies have been investigated to assess the relationship between the fertility related gene, *CYP19*, and reproduction in cow; however only scanty studies were performed on *CYP19* gene in buffalo. The present study was designed to isolate a fragment of *CYP19* gene containing exon 9 and to detect any polymorphism associated with infertility in Egyptian buffalo cows. *CYP19* gene fragment was isolated by using PCR technique and detected by the direct sequencing and single strand conformation polymorphism (SSCP). A fragment (locus) of *CYP19* gene with size of 446bp was successfully amplified. No SNP was detected in this locus as revealed by a monomorphic SSCP pattern observed in normal cyclic and anestrous animals. The results of SSCP were confirmed by nucleotide sequencing. Alignment of nucleotide sequences of this locus showed 100% identity with Indian water buffaloes (EF126034) and 99% identity with *Bos Taurus* (Z69249.1). The present study concluded that the sequence of *CYP19* is highly conserved between cattle and buffalo.

Keywords: CYP19, SSCP, PCR, Sequencing, Anestrus, Buffalo.

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1. INTRODUCTION

As a result to the gradual increase in the world population and shortage of water as well as decrease of green areas, it becomes necessary for developing countries to maximize the production of their native animals to guarantee a sustainable source for food of an animal origin. Among domestic animals, the water buffaloes (*Bubalus bubalis*), particularly the river buffalo, which is the main buffalo breed in Egypt. Indeed, selection of high fertile buffaloes on a genotype basis can improve the reproductive efficiency of the breed which will reflect on their productivity and on the national economics. One of the limiting

factors for quick genetic improvement in the buffalo population is poor reproduction. Field surveys on reproductive disorders revealed that anestrus was the most common single cause of infertility in buffaloes (Ashturkar et al., 1995; Singh and Sahni, 1995). The common genes of aromatase cytochromes P450 family are CYP19 and CYP21 (Kobylinska, 1994). The role of CYP19 is the conversion of androgens to estrogens, which is necessary to maintain the reproductive pattern in females (Evans et al., 2004; Ghai et al., 2012; Jędrzejczak et al., 2011; Murray et al., 2000). Detection of single nucleotide polymorphisms (SNPs) can be achieved

simply by many techniques including the single strand conformation polymorphism (SSCP) (Orita *et al.*, 1989), RFLP (Cotton, 1993; Youil *et al.*, 1995) and the denaturing gradient gel electrophoresis (Myers *et al.*, 1987; Sheffield *et al.*, 1989). The SSCP has been used for detecting the genetic mutations in humans (Orita *et al.*, 1989), rats (Pravenec *et al.*, 1992), cattle (Kirkpatrick, 1992; Raghavan, 2006), and in various bacteriological (Morohoshi *et al.*, 1991) and viral (Fugita *et al.*, 1992) systems. The SSCP followed by sequencing was implemented in the present study to discover polymorphism in CYP19 gene. Kumar *et al.* (2009) have analyzed CYP19 gene polymorphism by SSCP in 3 groups of buffaloes of different fertility performance, normal cyclic buffaloes, late matured and late maturing/true anestrus animals (Kumar *et al.*, 2009). Monomorphic SSCP patterns were observed in all three groups for exons 4, 5, 6 and 8. However, 4 allelic variants in coding exons 2, 9 and introns 3, 7 with unaltered protein sequence. Heterozygotic condition (C/ 82G) was observed in CYP19 exon 9 of late matured and true anestrus animals. Though there was a change from GG homozygote (control) to C/G heterozygote of late matured and true anestrus animals, the amino acid encoded by both the codons (CCC and CCG) is proline due to the degeneracy of the genetic code (Watson *et al.*, 2004). Indeed, previous studies have shown that the mutations in CYP19 gene cause aromatase deficiency that results in underdeveloped external genitalia and uteri, arrested follicles, and no corpora lutea (Grumbach and Auchus, 1999; Layman, 2002; Palter *et al.*, 2001). The aim of the present study was to isolate CYP19 gene locus containing exon 9 and to detect any polymorphisms associated with incidence of infertility problems in Egyptian buffalo cows.

2. MATERIAL AND METHODS

2.1. Animal source and grouping

The current study was conducted on a total of 180 buffalo cows and heifers selected from Buffalo Nucleus Herd, Animal Production Research Institute, Ministry of Agriculture. Heifers were naturally served for the first time when they reach 300 to 350 kg of body weight and/or 24 months of age. Buffalo cows should be dried off two months before the expected calving date, and they served not before two months after calving. Animals were assigned in two main groups: normal fertile and infertile due to anestrus. Buffalo cows with 5 successive calving will be grouped as normal fertile, heifers which didn't show oestrus over two years of age and buffaloes which didn't ovulate over sex months after calving was grouped as anoestrus.

2.2. Total DNA extraction

Blood samples were collected in EDTA-containing vacutainer tubes (kept in ice box) from jugular veins of 40 animals in each group. The genomic DNA was extracted from the leucocytes using Gene JET genomic DNA purification kit following the manufacturer protocol (Fermentas, #K0721). The concentration of total extracted DNA was spectrophotometrically determined at 230 and 260 nm using Nanodrop then stored at -20°C.

2.3. Polymerase chain reaction

The *CYP19* locus was amplified by PCR using primers (Table 1) designed by Primer 3.0 software based on the published sequences of Indian buffalo (GenBank accession number, EF126034). The PCR was carried out in a reaction volume of 50 µL, containing 4.0 µL DNA template (approximately 100 ng), 10 µl Dream Taq Green PCR master mix 5x (Fermentas, #K1071, European Union), 2.0 µL (10 µmol/L) forward primer, 2.0 µL (10 µmol/L) reverse primer, and 32 µL nuclease free water. PCR was performed by employing a PCR program as follows: Initial denaturation

step at 94°C for 2 min, then tubes were subjected to 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. As a negative control, tubes were prepared with water instead of DNA template. Then PCR products were resolved by electrophoresis on 1% agarose gel in 1X TAE, stained with ethidium bromide and visualized with UV light of Gel Documentation System (Biometra Biomedizinische Analytik, GmbH).

2.4. Single Strand Conformation Polymorphism (SSCP).

The procedure adapted in SSCP was described by Kumar et al (2009) with some modifications. The PCR products (5 µl) were mixed with 5 µl of SSCP dye (95% formamide, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue) in 0.2 ml PCR tubes, then incubated at 95°C for 10 min for denaturation and plunged in ice for 5 min to form single strand conformers then electrophoresed in a 12% polyacrylamide gel (ratio of acrylamide to bis-acrylamide was 39:1 [0.5 gram of bis-acrylamide and 19.5 gram of acrylamide was added to 50 ml distilled water and mixed well in water bath at 37°C till complete solubility, 5 ml 10% TBE (contained 108g Tris, 55g boric acid +40 ml 0.5M EDTA [37.22g EDTA in 150 ml of distilled water] and up to 1000 ml deionized water and mixed well), 12 ml of 20 % acrylamide to bis-acrylamide mix were added to 8 ml deionized water, 20 µl of TEMED (tetra methylene diamine) and finally 200 µl of 10% Ammonium persulfate was added to at a time and mixed well. The optimal polymerization time was about 4 h. The gel was pre electrophoresed at 160 V for 30 min with 0.5x TBE as electrode buffer. The conformers of PCR products were separated in the gel at 14–16°C using constant voltage of 160 V for 4 h. The DNA fragments in the gel were detected by 500ml of Ethidium bromide 0.5 µg/ml in 1x TBE for 10-30

minutes on a rocking platform for proper fixing of SSCP conformers in the gel. Then the gel was transferred gently in 500ml of sterile distilled water for de-staining. The fragment patterns were visualized on the UV Trans-illuminator and photographed by gel documentation system (UVDI Major Science, USA). After the polymorphism was detected, the PCR products of different electrophoresis patterns were purified and then sent to be sequenced.

2.5. DNA Sequencing

After getting purified PCR products (clones) with expected sizes, the clone was purified using PCR purification kit following the manufacturer protocol (Jena Bioscience # pp-201×s) to remove primer dimers, primers, nucleotides, proteins, salt, agarose, ethidium bromide and other impurities. The PCR products were sequenced in automated sequencer (Applied Biosystem, USA) using *CYP19* primers. The Sequences were analyzed using the Chromas Lite 2.1 program (http://technelysium.com.au/?page_id=13) and the identity of the sequenced PCR product was examined using Blast search against Genbank database of Indian buffalo and cattle (*Bos Taurus*) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The alignments, annotations and assembly of the sequences were performed using Geneious 4.8.4 software (<http://www.geneious.com/web/geneious/home>).

3. RESULTS

The PCR product with the expected size (446bp) was obtained as shown by 1% agarose gel electrophoresis photo (Fig. 1). Then, the resulting PCR products were purified using PCR purification technique. Subsequently, SSCP was carried out in order to detect the genotypes and to detect any SNPs. No polymorphism was detected in *CYP19* locus as revealed by presence of only

Table 1: Forward and reverse primers sequence for *CYP19* locus, annealing temperatures (Ta), size of PCR amplicon (bp) and its localization in the gene.

Gene	Primers		Ta (°C)	Size (bp)	Localization
	Forward (5-3)	Reverse(5-3)			
CYP19	TCTACGGAACAAGCAC AGGA	GGCACGCTCAGTTT TAAGGA	60	446	I8, E9, I9

I = Intron

E = Exon

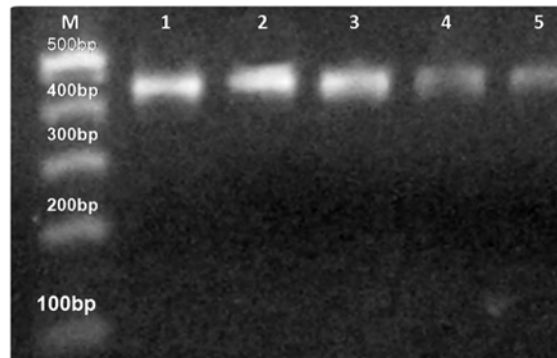


Fig.1. Ethidium bromide stained agarose gel of PCR products representing amplification of *CYP19* locus (lanes1-5) with size of 446 bp in Egyptian buffaloes. M represents 100bp ladder.

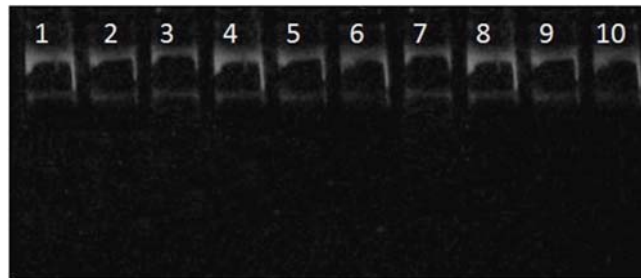


Fig.2. PCR-SSCP patterns of *CYP19* locus in Egyptian buffalo show the genotypes. One SSCP pattern was detected in this locus.

Isolation and sequencing of cyp19 gene (exon9) in Egyptian buffaloes

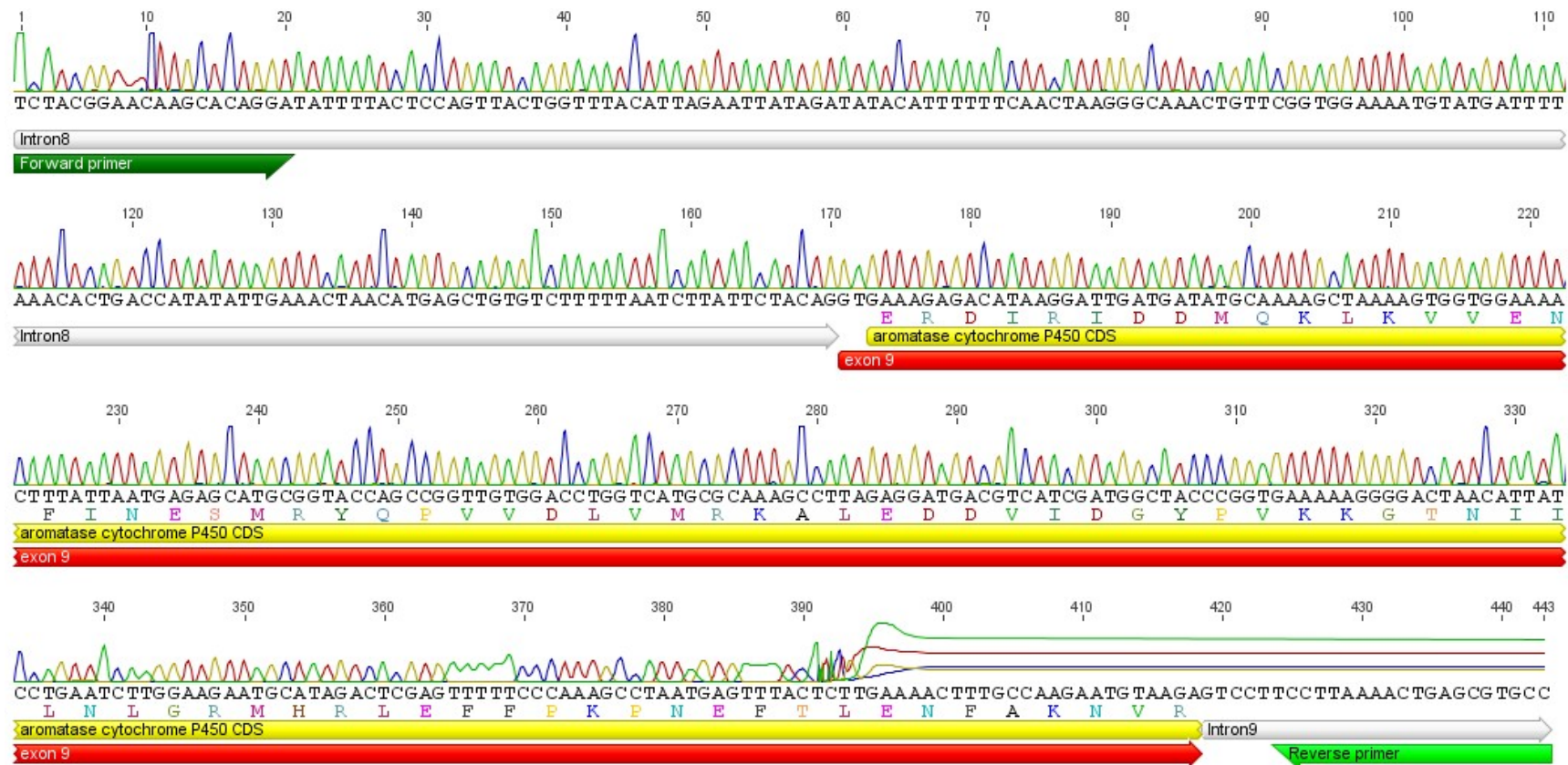


Fig.3. Nucleotide sequences of *CYP19* locus didn't show detected SNP. The corresponding amino acid sequences were shown below nucleotide sequences of exon 9.

Bubalus bubalis aromatase cytochrome P450 gene, exons 2, 3, 4, 6, 8, 9 and partial c

Sequence ID: [gb|EF126034.1](#) Length: 2620 Number of Matches: 1

Range 1: 2175 to 2617 [GenBank](#) [Graphics](#)

▼ Next Match ▲ Previous

Score	Expect	Identities	Gaps	Strand
819 bits(443)	0.0	443/443(100%)	0/443(0%)	Plus/Plus
Query 1	TCTACGGAACAAGCACAGGATATTTTACTCCAGTTACTGGTTTACATTAGAATTATAGAT	60		
Sbjct 2175	TCTACGGAACAAGCACAGGATATTTTACTCCAGTTACTGGTTTACATTAGAATTATAGAT	2234		
Query 61	ATACATTTTTTCAACTAAGGGCAAACCTGTTCCGGTGGAAAATGTATGATTTTAAACACTGA	120		
Sbjct 2235	ATACATTTTTTCAACTAAGGGCAAACCTGTTCCGGTGGAAAATGTATGATTTTAAACACTGA	2294		
Query 121	CCATATATTGAACTAACATGAGCTGTGTCTTTTAACTTATTCTACAGGTGAAAGAGA	180		
Sbjct 2295	CCATATATTGAACTAACATGAGCTGTGTCTTTTAACTTATTCTACAGGTGAAAGAGA	2354		
Query 181	CATAAGGATTGATGATATGCAAAAGCTAAAAGTGGTGGAAAACCTTTATTAATGAGAGCAT	240		
Sbjct 2355	CATAAGGATTGATGATATGCAAAAGCTAAAAGTGGTGGAAAACCTTTATTAATGAGAGCAT	2414		
Query 241	GCGGTACCAGCCGGTTGTGGACCTGGTCATGCGCAAAGCCTTAGAGGATGACGTCATCGA	300		
Sbjct 2415	GCGGTACCAGCCGGTTGTGGACCTGGTCATGCGCAAAGCCTTAGAGGATGACGTCATCGA	2474		
Query 301	TGGCTACCCGGTGAAAAAGGGGACTAACATTATCCTGAATCTTGAAGAATGCATAGACT	360		
Sbjct 2475	TGGCTACCCGGTGAAAAAGGGGACTAACATTATCCTGAATCTTGAAGAATGCATAGACT	2534		
Query 361	CGAGTTTTTCCCAAAGCCTAATGAGTTTACTCTTGAAAACCTTGCCAAGAATGTAAGAGT	420		
Sbjct 2535	CGAGTTTTTCCCAAAGCCTAATGAGTTTACTCTTGAAAACCTTGCCAAGAATGTAAGAGT	2594		
Query 421	CCTTCCTTAAACTGAGCGTGCC	443		
Sbjct 2595	CCTTCCTTAAACTGAGCGTGCC	2617		

Fig.4. Nucleotide sequences alignment of *CYP19* locus with Indian water buffaloes (EF126034) showed 100% identity.

Isolation and sequencing of cyp19 gene (exon9) in Egyptian buffaloes

Bos taurus partial cyp19 gene, exon 9

Sequence ID: [emb|Z69249.1|](#) Length: 601 Number of Matches: 1

Range 1: 107 to 549 [GenBank](#) [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
785 bits(425)	0.0	437/443(99%)	0/443(0%)	Plus/Plus
Query 1	TCTACGGAACAAGCACAGGATATTTTACTCCAGTTACTGGTTTACATTAGAATTATAGAT	60		
Sbjct 107	TCTACGGAACAAGCACAGGATATTTTACTCCAGTTATTGGTTTACATTAGAATTATAGAT	166		
Query 61	ATACATTTTTTCAACTAAGGGCAAACGTTCGGTGGAAAATGTATGATTTTAAACACTGA	120		
Sbjct 167	ATACATTTTTTCAACTAAGAGCAAACGTTCGTGGAAAATGTATGATTTTAAACACTGA	226		
Query 121	CCATATATTGAACTAACATGAGCTGTGTCTTTTAACTCTTATTCTACAGGTGAAAGAGA	180		
Sbjct 227	CCATATATTGACACTAACATGAGCTGTGTCTTTTAACTCTTATTCTACAGGTGAAAGAGA	286		
Query 181	CATAAGGATTGATGATATGCAAAAGCTAAAAGTGGTGGAAAACCTTTATTAATGAGAGCAT	240		
Sbjct 287	CATAAGGATTGATGATATGCAAAAGCTAAAAGTGGTGGAAAACCTTTATTAATGAGAGCAT	346		
Query 241	GCGGTACCAGCCGGTTGTGGACCTGGTCATGCGCAAAGCCTTAGAGGATGACGTCATCGA	300		
Sbjct 347	GCGGTACCAGCCCGTTGTGGACCTGGTCATGCGCAAAGCCTTAGAGGATGATGTCATCGA	406		
Query 301	TGGCTACCCGGTGAAAAAGGGGACTAACATTATCCTGAATCTTGAAGAATGCATAGACT	360		
Sbjct 407	TGGCTACCCGGTGAAAAAGGGGACTAACATTATCCTGAATCTTGAAGAATGCATAGACT	466		
Query 361	CGAGTTTTTCCCAAAGCCTAATGAGTTTACTCTTGAAAACCTTGCCAAGAATGTAAGAGT	420		
Sbjct 467	CGAGTTTTTCCCAAAGCCTAATGAGTTTACTCTTGAAAACCTTGCCAAGAATGTAAGAGT	526		
Query 421	CCTTCCTTAAACTGAGCGTGCC	443		
Sbjct 527	CCTTCCTTAAACTGAGCGTGCC	549		

Fig.5. Nucleotide sequences alignment of *CYP19* locus with *Bos Taurus* (Z69249.1) showed 99% identity.

one SSCP banding (monomorphic) pattern (Fig. 2). Sequencing was conducted to verify the results of SSCP. The sequences of *CYP19* locus in Egyptian buffaloes (submitted to GenBank with accession number KF976407) showed no polymorphism (Fig. 3). Nucleotide sequences alignment of *CYP19* locus showed 100% identity with Indian water buffaloes (EF126034) (Fig.4) and 99% identity with *Bos Taurus* (Z69249.1) (Fig.5).

4. DISCUSSION

Identification of genetic markers that can potentially improve reproduction and extension of the animal's productive life seems essential mainly for the breeding practice. Among the potential genetic markers analyzed in the present study is the *CYP19* gene which can play a role in predicting anestrus which is the most common single cause of infertility in buffaloes (Ashturkar *et al.*, 1995; Singh and Sahni, 1995), incorporated with the low level of ovarian estrogens (Hafez and Hafez, 2000). The key enzyme in estrogen biosynthesis is cytochrome P450 aromatase, the protein product of *CYP19* gene. The present study indicated the presence of an association between *CYP19* gene polymorphisms and reproduction of Egyptian buffalo-cows. Kumar *et al.* (2009) found a C82G SNP in coding region of *CYP19* exon9 in late matured and true anestrus animals (CG) as compared to GG control animals. Although, this SNP was in the coding region of exon9, the amino acid encoded by both the codons (CCC and CCG) is proline due to the degeneracy of the genetic code (Watson *et al.*, 2004). Thus, the aromatase amino acid sequence will be unaltered, retaining its activity. Therefore, this condition may not be related to anestrus (Kumar *et al.*, 2009). In contrast, we did not find such SNP or any other SNP in exon9 of all examined Egyptian buffaloes. This means that this SNP is not

conserved in *Bubalus bubalis*. Alignment of nucleotide sequences of *CYP19* locus in Egyptian buffaloes showed 100% identity with Indian water buffaloes (EF126034) and 99% identity with *Bos Taurus* (Z69249.1). This result indicates that the sequence of *CYP19* is highly conserved between cattle and buffalo. This is a preliminary study that provides the researchers with raw data, which could be used as a basis for further studies to associate other SNPs in *CYP19* with fertility in buffalo.

5. REFERENCES

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عزل وتسلسل الإكسون التاسع لجين *CYP19* في الجاموس المصري

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الملخص العربي

تم إجراء هذه الدراسة على عدد مائة وثمانين من الجاموس المصري النقي. وقد تم الحصول على هذه الحيوانات من محطه النطاف بمحلة موسى بكفر الشيخ. وقد تم تحديد الطفرة الموجودة في الإكسون التاسع لجين *CYP19* والتي لم تؤد إلى أي تغيير في النيوكليوتيدات في هذا الموقع من الجين. لقد تمت دراسات عديدة على الارتباط بين الجين المتعلق بالخصوبة *CYP19* والتناسل في الأبقار ولكن أجريت فقط دراسات ضئيلة على هذا الجين في الجاموس، لذلك أجريت هذه الدراسة لعزل جزء من جين *CYP19* تحتوي الإكسون التاسع والتعرف على أي طفرات مرتبطة بعدم الشياح في إناث الجاموس المصري. لتحقيق ذلك تم عزل جزء من جين *CYP19* باستخدام طريقة التفاعل البلمري المتسلسل ثم تم الكشف عن الطفرات بالتسلسل المباشر وطريقة SSCP. تم عزل جزء من جين ال *CYP19* يصل حجمه إلى 446 زوج من القواعد النيتروجينية ولكن لم يتم الكشف عن طفرات في هذا الجزء ولقد تحقق ذلك بظهور طرز أحادي الشكل في الحيوانات الطبيعية والتي لم يتم فيها الشياح. للتأكيد على هذه النتائج تم عمل تسلسل للقواعد النيتروجينية. مقارنة تتابع النيوكليوتيدات في هذا الموقع من الجين أثبت تماثل بنسبة 100 % مع الجاموس الهندي و99% مع الأبقار. هذه النتائج تدل على أن التسلسل لجين *CYP19* عالي التشابه بين الأبقار والجاموس. هذه دراسة مبدئية تمد الباحثين بالبيانات الخام والتي يمكن أن تستخدم كقاعدة لدراسات إضافية لربط الطفرات في نيوكليوتيدة أحادية في جين *CYP19* بالخصوبة في الجاموس.

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